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Improving adenovirus vector-mediated RNAi efficiency by lacking the expression of virus-associated RNAs

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ABSTRACT

Several studies have reported that short hairpin RNA (shRNA)-mediated RNA interference (RNAi) was competitively inhibited by the expression of adenovirus (Ad)-encoded small RNAs (VA-RNAs), which are expressed from a replication-incompetent Ad vector, as well as a wild-type Ad; however, it remained to be clarified whether an shRNA-expressing Ad vector-mediated knockdown was inhibited by VA-RNAs transcribed from the same Ad vector genome. In this study, we demonstrated that a lack of VA-RNA expression from the Ad vector leads to an increase in knockdown efficiencies of Ad vector-mediated RNAi. In the cells transduced with a first-generation Ad vector (FG-Ad) expressing shRNA (FG-Ad-shRNA), the copy numbers of shRNA and VA-RNAs incorporated into the RNA-induced silencing complex (RISC) was comparable. In contrast, higher amounts of shRNA were found in the RISC when the cells were transduced with an shRNA-expressing helper-dependent Ad (HD-Ad) vector, in which all viral genes, including VA-RNAs, were deleted (HD-Ad-shRNA), compared with FG-Ad-shRNA. HD-Ad vectors expressing shRNA against luciferase and p53 showed 7.4% and 37.3% increases in the knockdown efficiencies compared to the corresponding FG-Ad-shRNA, respectively, following *in vitro* transduction. Furthermore, higher levels of knockdown efficiencies were also found by the transduction with shRNA-expressing Ad vectors lacking VA-RNA expression (AdΔVR-shRNA) than by transduction with FG-Ad-shRNA. These results indicate that VA-RNAs expressed from an Ad vector inhibit knockdown by the shRNA-expressing Ad vector and that HD-Ad-shRNA and AdΔVR-shRNA are a powerful framework for shRNA-mediated knockdown.

1. Introduction

RNA interference (RNAi) has become one of the most important techniques for both gene-function analysis in basic research and silencing of disease-related genes in clinical applications. In addition to chemically synthesized small interfering RNA (siRNA), vectors expressing short hairpin RNA (shRNA) under the control of RNA polymerase III promoters and RNA polymerase II promoters are widely used to induce RNAi in mammalian cells (Giering et al., 2008; Scherer and Rossi, 2003; Tuschl, 2002). The shRNA-based systems for RNAi have various advantages. First, shRNA expression can be controlled by various types of promoters. Not only the ubiquitous U6 and H1 promoters but also tissue-specific promoters, including the liver-specific apolipoprotein E enhancer/human α1-antitrypsin promoter, are available for shRNA-mediated knockdown (Giering et al., 2008). Second, an shRNA expression cassette is efficiently delivered to targeted cells not only by non-viral vectors, but also by viral vectors, such as adenovirus (Ad), lentivirus, and adeno-associated virus vectors. An shRNA-expressing vector can be selected, depending on the situation, since each shRNA-expressing vector has distinct gene-transfer properties and activities (Grimm and Kay, 2007).

Among the various types of vectors for shRNA expression, Ad vectors are highly promising as potential shRNA-expressing vectors because of their superior transduction properties (Benihoud et al., 1999; Koizumi et al., 2007; Xu et al., 2005). We and other groups previously demonstrated that Ad vector-mediated RNAi has great potential for use as an shRNA-expressing vector in a variety of applications (Hosono et al., 2005; Hosono et al., 2004; Mizuguchi et al., 2007; Motegi et al., 2011). However, it was reported that virus-associated RNA (VA-RNA) I (a major species) and VA-RNA II (a minor species), which are small RNAs transcribed from the

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Ad genome, inhibit shRNA-mediated RNAi by competition for key components involved in the RNAi pathway (Andersson et al., 2005; Aparicio et al., 2006; Lu and Cullen, 2004; Xu et al., 2007). VA-RNA I and II are about 160 nucleotide-long non-coding RNAs encoded in the Ad genome (bp 10620–10779 and 10876–11038, respectively) (Bhat et al., 1983; Fowlkes and Shenk, 1980; Machitani et al., 2011b; Mathews and Shenk, 1991). After transcription, the VA-RNAs are transported to the cytoplasm by the interaction between their terminal stems and exportin-5, and then processed by an RNase III-type enzyme dicer, resulting in the production of VA-RNA-derived microRNAs (miRNAs) (mivaRNAs) (Andersson et al., 2005; Lu and Cullen, 2004; Xu et al., 2007). After being processed by the dicer, the mivaRNAs are incorporated into an RNA-induced silencing complex (RISC). shRNA is also processed into siRNA through the same pathway. Since VA-RNAs are also expressed from a replication-incompetent first-generation Ad (FG-Ad) vector (Yamaguchi et al., 2010), VA-RNAs transcribed from FG-Ad vectors could inhibit knockdown efficiency of FG-Ad vector-mediated RNAi by competitive inhibition of shRNA processing in several steps, *i.e.*, transport by exportin-5, digestion by the dicer, and incorporation into RISC. Based on these findings, we hypothesized that shRNA-expressing Ad vectors lacking VA-RNA expression exhibit more effective RNAi compared with FG-Ad-shRNA; however, there have been no studies that directly evaluate the inhibitory effects of VA-RNAs transcribed from the Ad vector genome on shRNA-expressing Ad vector (Ad-shRNA)-mediated knockdown.

In the present study, we prepared an shRNA-expressing helper-dependent Ad (HD-Ad) vector, in which almost all viral coding sequences, including VA-RNAs (Palmer and Ng, 2005), were deleted (HD-Ad-shRNA). Titers of HD-Ad-shRNA and FG-AD-shRNA were properly evaluated under the same condition by measuring the expression levels of the green fluorescence protein (GFP) gene, which was inserted into the Ad vector genome. HD-Ad-shRNA showed significantly higher levels of knockdown efficiencies than FG-Ad-shRNA following *in vitro* transduction. Furthermore, shRNA-expressing Ad vectors lacking VA-RNA expression (AdΔVR-shRNA), which are designed to lack only VA-RNA-expression through specific deletion of the transcriptional control elements of VA-RNA, also showed more efficient knockdown than FG-Ad-shRNA.

2. Materials and methods

2.1. Cells

HEK293 (a transformed embryonic kidney cell line), SK HEP-1 (a human hepatoma cell line) and VR293 cells (HEK293 cells inducibly expressing VA-RNA I) were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml). WI38 (a normal human lung diploid fibroblast) and 116 cells (Cre recombinase-expressing cells for packaging of HD-Ad vectors) were cultured with Minimum Essential Medium supplemented with 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml).

2.2. Plasmids and virus

HD-Ad vector plasmids, pSTK129-shLuc-GFP, were constructed to express shRNA against firefly luciferase as follows. pHM18-GFP, containing a GFP expression cassette driven by a cytomegalovirus (CMV) promoter, was previously constructed using the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) and a shuttle plasmid, pHM18 (Yamaguchi et al., 2010). pHM18-GFP was digested with *Xba*I/*Sph*I, and ligated with the *Xba*I/*Sph*I fragment of pHM5-ihU6-Lu (Mizuguchi et al., 2007) after an *Sma*I site of pHM5-ihU6-Lu had been changed into an *Xba*I site by using *Xba*I phosphorylated linkers (New England Biolabs, Beverly, MA), resulting in pHM18-ihU6-Lu-GFP. pHM18-ihU6-Lu-GFP was digested with *Not*I, and the *Not*I fragments were inserted into the *Not*I site of pSTK129 (kindly provided by Dr. Ng Philip) (Palmer and Ng, 2003), resulting in pSTK129-shLuc-GFP. Then, pSTK129-shLuc-GFP was digested with *Pme*I, and the liberated viral genome was transfected into 116 cells expressing Cre recombinase. The HD-Ad vectors were amplified and purified as described previously (Palmer and Ng, 2003). Helper-virus contamination of the HD-Ad vectors was assessed by quantitative real-time PCR, and the level of contamination was estimated to be less than 0.3%. HD-Ad-shp53 was similarly prepared as described above.

The FG-Ad and AdΔVR vectors were constructed by means of an improved *in vitro* ligation method described previously (Mizuguchi and Kay, 1998; Mizuguchi and Kay, 1999; Mizuguchi et al., 2001b; Sakurai et al., 2005) as follows. pHM5 (Mizuguchi and Kay, 1999) was digested with *Sph*I/*Kpn*I, and ligated with the *Sph*I/*Kpn*I fragment of pHM18-ihU6-Lu-GFP, resulting in pHM5-ihU6-Lu-GFP. pHM5-ihU6-Lu-GFP was digested with I-*Ceu*I/PI-*Sce*I, and then ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 (Mizuguchi and Kay, 1998) or pAdHM4ΔVR (Machitani et al., 2011a), resulting in pAdHM4-shLuc-GFP and pAdHM4ΔVR-shLuc-GFP, respectively. To generate FG-Ad vector particles, pAdHM4-shLuc-GFP was digested with *Pac*I to release the recombinant viral genome, and then transfected into HEK293 cells, resulting in FG-Ad-shLuc. AdΔVR-shRNA was prepared as previously described using VR293 cells (Machitani et al., 2011a), instead of HEK293 cells. FG-Ad-shp53 was similarly prepared as described above. The amplified Ad vectors were purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80 °C (Mizuguchi and Kay, 1998). The virus particles (VP) were determined by a spectrophotometrical method (Mizuguchi et al., 2001a). Biological titers, which were based on GFP transduction activity, were determined by serial titration of the vector stocks on SK HEP-1 cells, followed by fluorescence-activated cell sorter (FACS) analysis for GFP-positive cells (Machitani et al., 2011a).

2.3. Preparation of SK HEP-1 transformants expressing luciferase (SK HEP1-Luc)

SK HEP-1 cells were transduced with a lentivirus vector, LV-RVLuP, expressing the Venus fluorescent protein and firefly luciferase as a fusion protein linked with a 2A-self-cleaving peptide (see *Supplementary Information*). The transduced cells expressing the Venus fluorescent protein were sorted by using a FACSAria™ cell sorter (BD Bioscience, San Jose, CA) (Supplemental Fig. 4). The resulting cells were designated SK HEP-1-Luc cells. SK HEP-1-Luc cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml).

2.4. Determination of small RNA levels incorporated into RISC

RNA incorporated into the RISC was isolated following transduction with Ad vectors using a microRNA Isolation Kit, human argonaute 2 (Ago2) (Wako, Osaka, Japan) according to the manufacturer's instructions. Briefly, Ago2, which is the main component of RISC, was immunoprecipitated by anti-Ago2 antibody to recover RNA in the RISC. After immunoprecipitation, the Ago2-small RNA complex was eluted using elution solution, and small RNA was isolated from the complex using Isogen (Nippon Gene, Tokyo, Japan). Copy numbers of siRNA and VA-RNAs in the isolated RNA (160 ng) were determined by quantitative RT-PCR using Mir-X™ miRNA first-strand synthesis and SYBR® qRT-PCR (Clontech-Takara, Kyoto, Japan) and primers specific for the siLuc, sip53, and

mivaRNAI. The sequences of the primers were as follows: siLuc, 5′-atttcgaagtactcagcgt-3′; sip53, 5′-gtagattaccactggagtc-3′; mivaRNAI, 5′-gacaacgggggagtgctcc-3′.

2.5. Real time RT-PCR analysis

Whole cell lysates for quantitative RT-PCR were prepared from the cells transduced with Ad vectors using a CellAmp® direct RNA Prep Kit (Takara-Bio, Otsu, Japan). After cell lysis, luciferase and p53 mRNA levels were determined by quantitative RT-PCR using a One Step SYBR® PrimeScript® RT-PCR Kit II (Takara-Bio, Otsu, Japan). The primers used for quantitative RT-PCR were as follows: luciferase-F, 5′-tcctatgattatgtccggttatgtaaa-3′; luciferase-R, 5′-tgtagccatccatccttgtcaa-3′; p53-F, 5′-ccgcagtcagatcctagcg-3′; p53-R, 5′-aatcatccattgcttgggacg-3′.

2.6. Luciferase assay

SK HEP-1-Luc cells were transduced with shLuc-expressing Ad vectors. After a 36-h incubation, luciferase activity in the cells was determined using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan).

2.7. Western blot analysis

Whole-cell extracts were prepared by suspension of the cells in lysis buffer (20 mM HEPES [pH 7.5], 1% Triton X-100, 2 mM EGTA, 10% glycerol, 5 mM dithiothreitol, 4 mM phenylmethylsulfonyl fluoride) 48 h after transduction. The protein content was measured with a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Protein samples (15 μg) were electrophoresed on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing conditions, followed by electrotransfer to PVDF membranes (Millipore, Bedford, MA). After blocking with 4% skim milk prepared in TBS-T (tween-20, 0.1%), the membrane was incubated with anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation in the presence of horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA). The membrane was developed with a chemiluminescence kit (ECL Plus Western blotting detection system; Amersham Biosciences, Piscataway, NJ), and then the signals were read with an LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

2.8. Statistical analysis

Statistical significance was determined using Student's *t*-test. Data are presented as the means ± S.D.

3. Results

3.1. Construction of shRNA-expressing FG-Ad and HD-Ad vectors

First, in order to compare the knockdown efficiencies of FG-Ad and HD-Ad vectors expressing shRNA, we constructed the FG-Ad and HD-Ad vectors carrying a U6 promoter-driven shRNA-expression cassette to induce knockdown of target genes (luciferase and human p53 genes). In addition, a CMV promoter-driven GFP-expression cassette was incorporated into the vector genome to precisely determine the titers of these Ad vectors (Fig. 1). We demonstrated that the transduction efficiencies by these Ad vectors at the same multiplicity of infection (MOI) were comparable (Supplemental Figs. 1–3). SK HEP-1 cells were more susceptible to Ad vector-mediated transduction than WI38 cells (Supplemental Figs. 1 and 2).

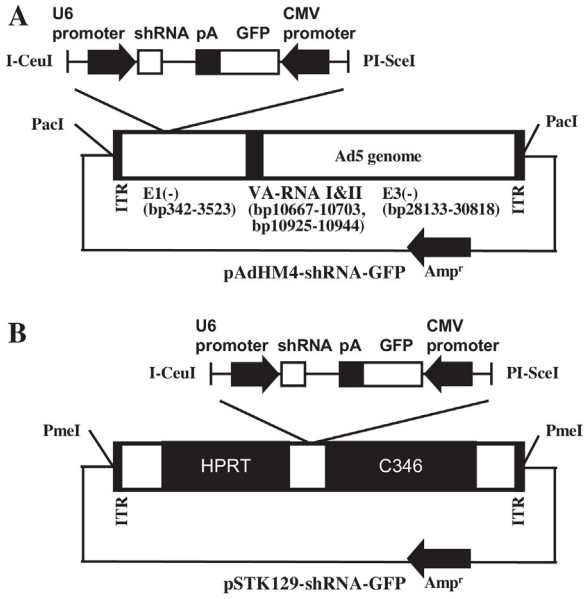

Fig. 1. Vector plasmids for the generation of FG-Ad-shRNA and HD-Ad-shRNA. The structure of the vector plasmids (A) pAdHM4-shRNA-GFP (for FG-Ad-shRNA) and (B) pSTK129-shRNA-GFP (for HD-Ad-shRNA). pA: bovine growth hormone (BGH) poly A sequence; ITR: inverted terminal repeat; Amp^r: ampicillin resistance gene; HPRT: intron region of human genomic HPRT stuffer sequence; C346: cosmid C346 human genomic stuffer sequence.

3.2. Copy numbers of siRNA and VA-RNA in the RISC

Next, in order to examine whether incorporation of siRNA which was processed from the shRNA into RISC was inhibited by VA-RNAs, SK HEP-1 cells constitutively expressing firefly luciferase (SK HEP-1-Luc cells) were transduced with a FG-Ad vector expressing shRNA against the luciferase gene (shLuc) (FG-Ad-shLuc), and then, the copy numbers of siLuc (the processed form of shLuc) and mivaRNAI in the RISC were determined by real-time RT-PCR. The analysis demonstrated that the copy numbers of siLuc and mivaRNAI in the RISC were almost comparable following the transduction with FG-Ad-shLuc at all the MOIs (Fig. 2A). On the other hand, when the cells were transduced with an HD-Ad vector expressing shLuc (HD-Ad-shLuc), a significantly higher amount of siLuc was found in the RISC, compared with FG-Ad-shLuc (Fig. 2B). Transduction with HD-Ad-shLuc resulted in an approximately 5-fold higher amount of siLuc in the RISC than that with FG-Ad-shLuc at the MOIs of 1, 3, and 10 (Fig. 2B). A significantly higher amount of sip53, which is processed from an shRNA against human p53 (shp53), was also found in the RISC for an HD-Ad vector expressing shp53 (HD-Ad-shp53), compared with an FG-Ad vector expressing shp53 (FG-Ad-shp53), in WI38 cells (Fig. 2C). These results suggest that the competition between shRNA and VA-RNAs, both of which are transcribed from the same Ad vector genome, in the processing steps leads to the lower copy numbers of siRNA in the RISC when transduced with FG-Ad-shRNA, compared with HD-Ad-shRNA.

3.3. Efficient knockdown of target genes by HD-Ad-shRNA

Next, to evaluate the effect of VA-RNAs on the knockdown efficiencies of shRNA-expressing Ad vectors, SK HEP-1-Luc cells were transduced with FG-Ad-shLuc or HD-Ad-shLuc at an MOI of 1, 3, or 10. In this experiment, we confirmed that a FG-Ad vector expressing shRNA against the other gene (p53 gene) (FG-Ad-shp53) did not knockdown luciferase expression (Supplemental Fig. 4). HD-Ad-shLuc mediated an approximately 10% increase in the efficiency of knockdown of luciferase mRNA at the MOIs of 3 and 10 (Fig. 3A)

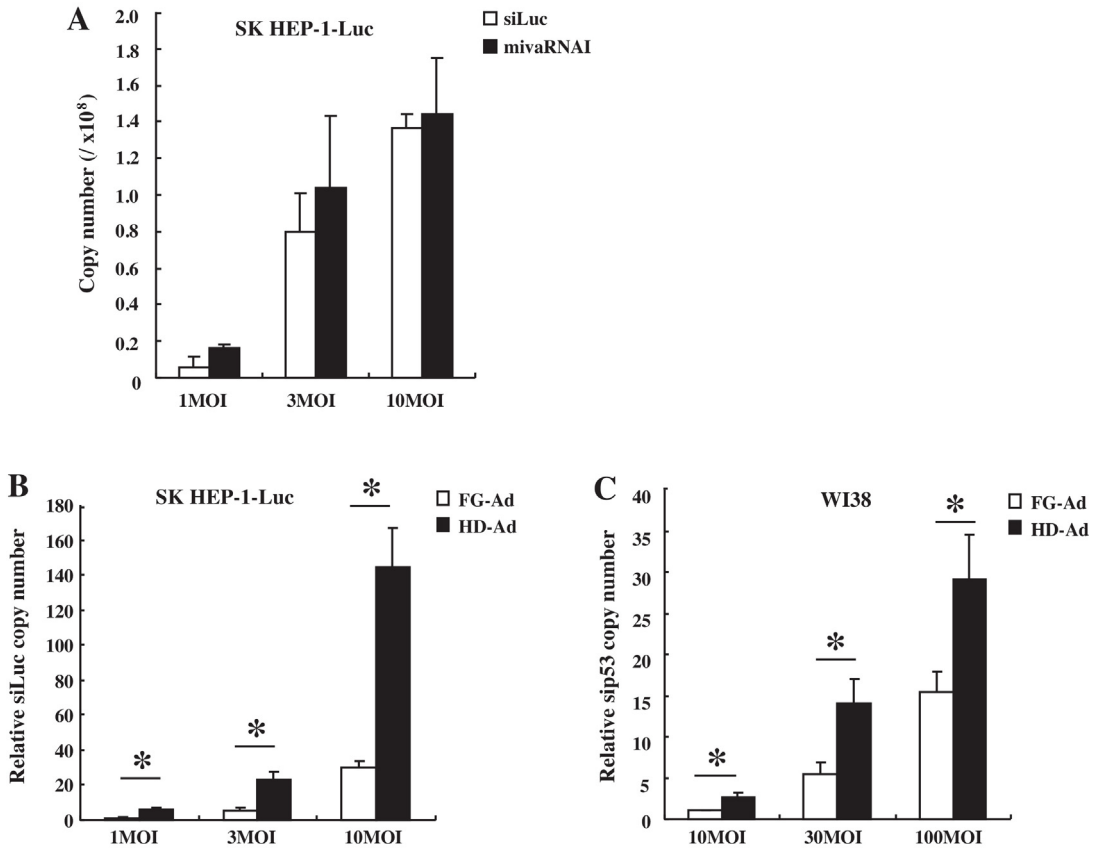

Fig. 2. Comparison of small RNA levels incorporated into RISC.
(A) Copy numbers of siLuc (the processed form of shLuc) and mivaRNAI in the RNA-induced silencing complex (RISC) recovered from SK HEP-1-Luc cells following transduction with FG-Ad-shLuc-GFP. (B) Copy numbers of siLuc and mivaRNAI in the RISC recovered from SK HEP-1-Luc cells following transduction with HD-Ad-shLuc-GFP. SK HEP-1-Luc cells were transduced with FG-Ad-shLuc or HD-Ad-shLuc at a multiplicity of infection (MOI) of 1, 3 or 10. Total RNA was recovered from the RNA-induced silencing complex (RISC) 36 h after transduction with Ad vectors. Copy numbers of siLuc and mivaRNAI were determined by quantitative RT-PCR. The copy number of siLuc in the RISC following transduction with FG-Ad-shLuc at an MOI of 1 was normalized to 1. (C) Copy numbers of sip53 in the RISC recovered from WI38 cells following transduction with FG-Ad-shp53 or HD-Ad-shp53. WI38 cells were transduced with FG-Ad-shp53 or HD-Ad-shp53 at an MOI of 10, 30 or 100. Total RNA was isolated from the RISC 48 h after transduction, and copy numbers of sip53 were determined by quantitative RT-PCR. The copy number of sip53 in the RISC following transduction with FG-Ad-shp53 at an MOI of 10 was normalized to 1. These experiments were repeated at least three times, and representative data are shown. All the data are expressed as means ± S.D. ($n = 3$). $^*p < 0.01$.

and in the efficiency of knockdown of luciferase activity levels at all MOIs (Fig. 3B), compared with FG-Ad-shLuc. Significantly higher knockdown efficiencies were also found for HD-Ad-shp53 than FG-Ad-shp53 in WI38 cells (Fig. 4A and B). Surprisingly, the p53-knockdown efficiencies were more greatly improved by the use of an HD-Ad vector, compared with the luciferase-knockdown efficiencies, although the increases in the copy numbers of siLuc in the RISC were larger than those of sip53 by the use of an HD-Ad vector. A 37% increase in the p53-knockdown efficiency was observed when the WI38 cells were transduced with HD-Ad-shp53 at an MOI of 100, compared with FG-Ad-shp53 (Fig. 4A). Significantly more efficient knockdown of p53 was also found for HD-Ad-shp53 than FG-Ad-shp53 in SK HEP-1 (Supplemental Fig. 5). These results indicate that HD-Ad-shRNA shows higher levels of knockdown efficiencies than FG-Ad-shRNA.

3.4. Efficient knockdown of target genes by AdΔVR-shRNA

As described above, HD-Ad-shRNA showed higher levels of knockdown efficiencies than FG-Ad-shRNA; however, it is unclear whether the deletion of VA-RNAs really contributed to the enhanced knockdown efficiencies of HD-Ad-shRNA, because HD-Ad vectors are devoid of all viral genes, including VA-RNAs (Palmer and Ng, 2005). Therefore, in order to further demonstrate that VA-RNAs inhibit shRNA-mediated knockdown, the knockdown efficiencies of shRNA-expressing AdΔVR vectors were examined (Fig. 5A). AdΔVR vectors, which were previously developed by our group, lack VA-RNA expression due to deletion of the transcriptional control elements for VA-RNA expression (Machitani et al., 2011a). We confirmed that the transduction efficiencies of FG-Ad- and AdΔVR-shLuc at the same MOIs were comparable (Supplemental Fig. 3). Real-time RT-PCR analysis demonstrated that an approximately 2-fold higher amount of siLuc was found in the RISC following transduction with AdΔVR-shLuc, compared with FG-Ad-shLuc at an MOI of 10 (Fig. 5B). Furthermore, AdΔVR-shLuc showed 9.7% higher knockdown efficiencies than FG-Ad-shLuc at an MOI of 10 (Fig. 5C and D), suggesting that the deletion of VA-RNAs led to an enhancement in the knockdown efficiency of shRNA-expressing Ad vectors.

4. Discussion

The aim of this study is to examine whether an shRNA-expressing Ad vector-mediated knockdown was inhibited by VA-RNAs transcribed from the same Ad vector genome. So far, previous studies have reported the suppression of shRNA-mediated RNAi by VA-RNAs (Andersson et al., 2005; Lu and Cullen, 2004); however, shRNA and VA-RNAs were expressed separately from the two different plasmids in these studies. In this study, we constructed shRNA-expressing HD-Ad vectors and AdΔVR vectors,

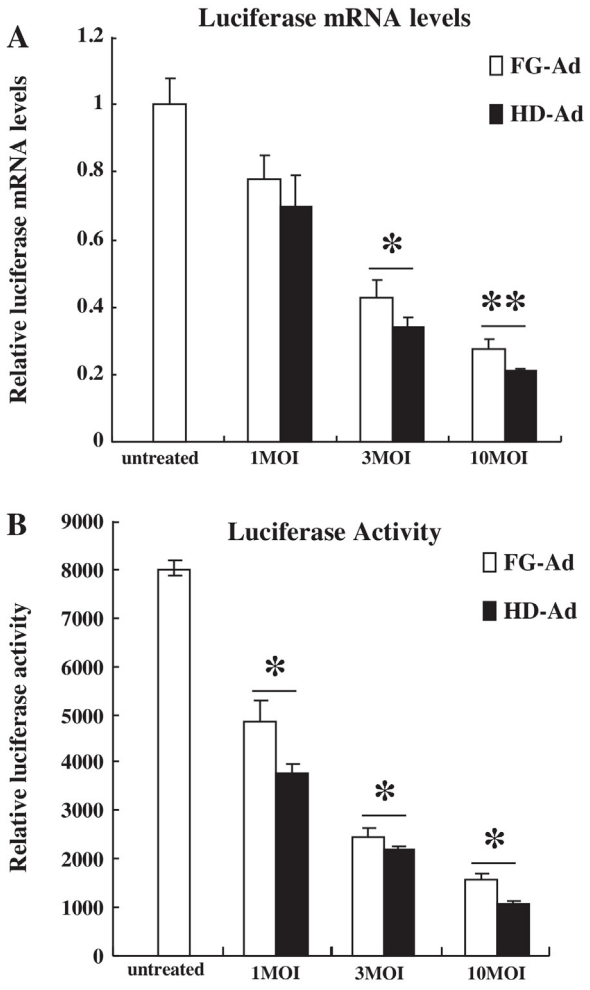

Fig. 3. Knockdown efficiencies of FG-Ad-shLuc and HD-Ad-shLuc in SK HEP-1-Luc cells.
Luciferase mRNA levels (A) and luciferase activity (B) following transduction with FG-Ad-shLuc or HD-Ad-shLuc in SK HEP-1-Luc cells. SK HEP-1-Luc cells were transduced with FG-Ad-shLuc or HD-Ad-shLuc at a multiplicity of infection (MOI) of 1, 3 or 10. After a 36-h incubation, mRNA levels of the luciferase gene and luciferase activity were determined. $^{*}p < 0.05$, $^{**}p < 0.01$.

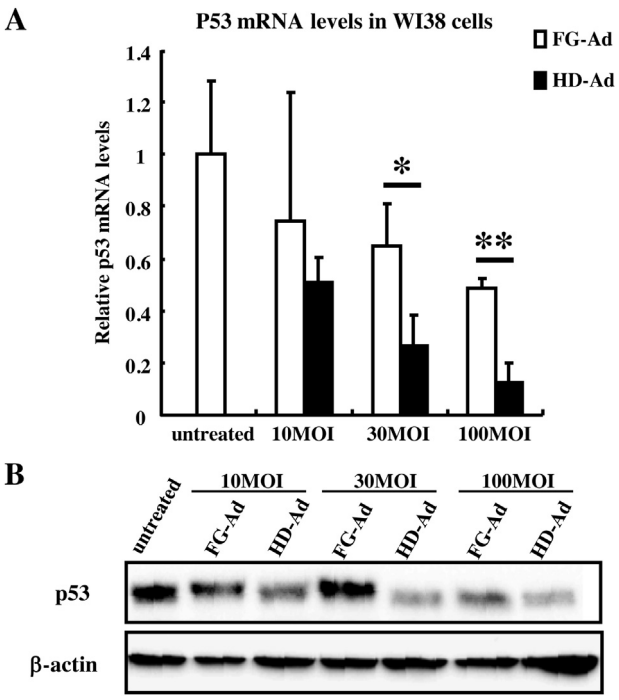

Fig. 4. Knockdown efficiencies of FG-Ad-shp53 and HD-Ad-shp53 in WI38 cells.
(A) p53 mRNA levels following transduction with FG-Ad-shLuc or HD-Ad-shLuc in WI38 cells. Cells were transduced with FG-Ad-shp53 or HD-Ad-shp53 at an MOI of 10, 30 or 100. After a 48-h incubation, mRNA levels of the p53 gene were determined by quantitative RT-PCR. All the data are expressed as the means ± S.D. ($n = 4$). (B) p53 protein levels following transduction with FG-Ad-shLuc or HD-Ad-shLuc in WI38 cells. WI38 cells were transduced with FG-Ad-shp53 or HD-Ad-shp53 at an MOI of 10, 30 or 100. After a 48-h incubation, Western blot analysis for p53 was performed. These experiments were repeated at least three times, and representative data are shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

which do not express VA-RNAs, and evaluated the knockdown efficiencies. shRNA-expressing HD-Ad vectors and AdΔVR vectors exhibited higher knockdown efficiencies than shRNA-expressing FG-Ad vectors in this study. VA-RNAs and miRNA/shRNA shares the common processing steps, including export into cytosol, digestion by dicer, and loading into RISC. Lu et al. demonstrated that VA-RNAs competitively inhibit miRNA biogenesis (Lu and Cullen, 2004). In addition, VA-RNAs have recently been demonstrated to suppress dicer expression by competitive binding for exportin-5 between dicer mRNA (Bennasser et al., 2011). Therefore, HD-Ad- and AdΔVR-shRNA do not inhibit miRNA processing due to the lack of VA-RNA expression. This property is highly crucial, especially for shRNA-expressing Ad vectors. Grimm et al. reported that overexpression of shRNA in the liver resulted in suppression of miRNA processing, leading to severe hepatotoxicity (Grimm et al., 2006). Expression of both VA-RNAs and shRNA might inhibit miRNA processing more severely than shRNA alone.

The present study suggests that HD-Ad-shRNA and AdΔVR-shRNA have greater potential to efficiently induce RNAi effects, compared with FG-Ad-shRNA. The application of HD-Ad- and AdΔVR-shRNA will be helpful for decreasing the viral dose required to achieve an RNAi-mediated therapeutic effect. Furthermore, HD-Ad-shRNA eliminates the possibility of leaky expression of the viral gene and subsequent toxic effects, because HD-Ad vectors delete almost all of the viral coding sequence. These characteristics could contribute to a reduction of the immunological side effects caused by the Ad vector itself (Chen et al., 2003; Jooss and Chirmule, 2003; Koizumi et al., 2007; Liu and Muruve, 2003; Muruve, 2004; Sakurai et al., 2008; Xu et al., 2005; Yamaguchi et al., 2007).

As described above, HD-Ad and AdΔVR vectors are superior to FG-Ad vectors in the shRNA-mediated knockdown efficiencies; however, the expression of VA-RNAs from the plasmids resulted in higher levels of reduction in the shRNA-mediated knockdown efficiencies, compared with the differences in the knockdown efficiencies between FG-Ad vectors and HD-Ad vectors or AdΔVR vectors shown in the present study. Andersson et al. reported that knockdown efficiencies by plasmids expressing shLuc were attenuated from about 80% to 30% by co-transfection with plasmids expressing VA-RNAs (Andersson et al., 2005). Similar results were shown by Lu et al. (Lu and Cullen, 2004) This is probably because the expression levels of VA-RNAs per plasmid-transfected cell were higher than those per Ad vector-transduced cell, although the numbers of VA-RNA-expressing cells were lower for the plasmid transfection than for transduction with an Ad vector.

There was no apparent correlation between the knockdown efficiencies of shRNA-expressing Ad vectors and the copy numbers of the siRNA in the RISC. About 5-fold higher amounts of siLuc were found in the RISC following transduction with HD-Ad-shLuc, compared with FG-Ad-shLuc; however, HD-Ad-shLuc showed only a 7.4% increase in the knockdown efficiencies compared with FG-Ad-shLuc (Fig. 3A and B). Although AdΔVR-shLuc mediated about 2-fold elevation in the copy number of siLuc in the RISC at MOI of

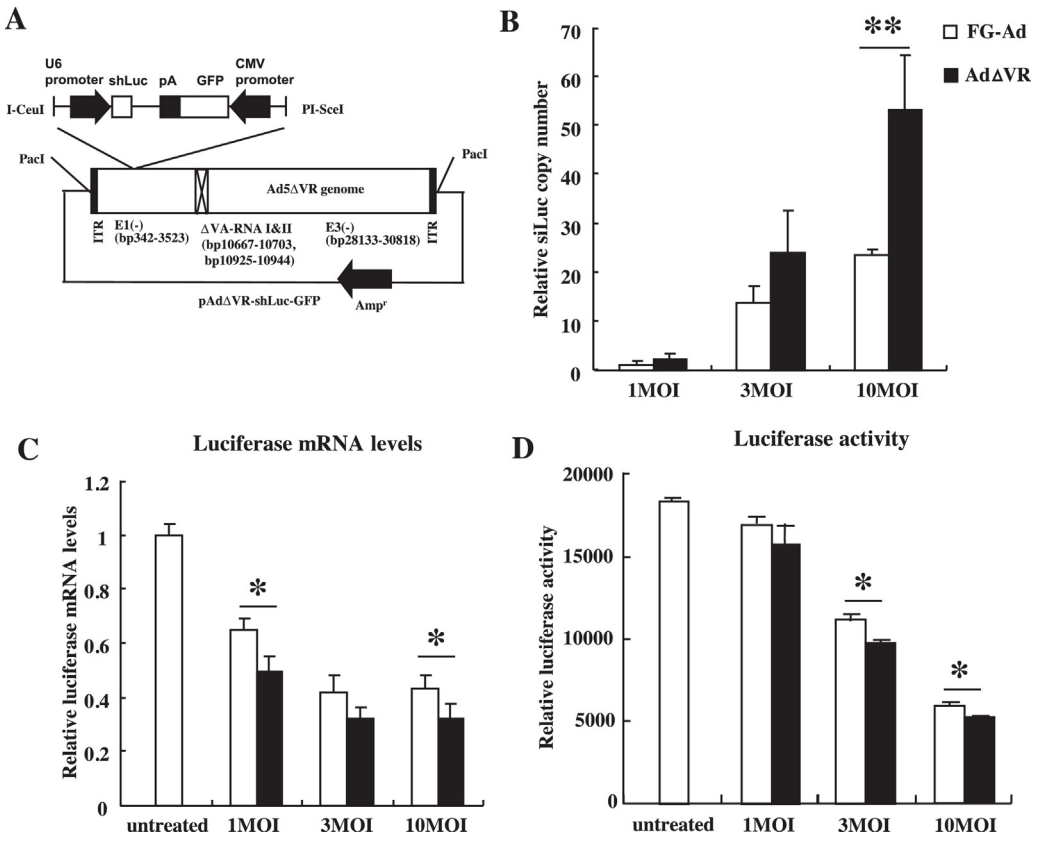

Fig. 5. Knockdown efficiencies of FG-Ad-shLuc and AdΔVR-shLuc.
(A) The structure of the vector plasmid, pAdΔVR-shLuc-GFP. pA: bovine growth hormone (BGH) pA sequence; ITR: inverted terminal repeat; Amp^r: ampicillin resistance gene. (B) Copy numbers of siLuc in the RISC recovered from SK HEP-1-Luc cells following transduction with FG-Ad-shLuc or AdΔVR-shLuc. SK HEP-1-Luc cells were transduced with FG-Ad-shLuc or AdΔVR-shLuc at a multiplicity of infection (MOI) of 1, 3 or 10. After a 36-h incubation, total RNA was extracted from the RISC. The copy numbers of siLuc were determined by quantitative RT-PCR. The copy number of siLuc in the RNA-induced silencing complex (RISC) following transduction with FG-Ad-shLuc at an MOI of 1 was normalized to 1. The data are expressed as the means ± S.D. ($n = 3$). (C, D) Luciferase mRNA levels (C) and luciferase activity (D) following transduction with FG-Ad-shLuc or AdΔVR-shLuc. SK HEP-1-Luc cells were transduced with FG-Ad-shLuc or HD-Ad-shLuc at an MOI of 1, 3 or 10. After a 36-h incubation, mRNA levels of the luciferase gene and luciferase production were determined. These experiments were repeated at least three times, and representative data are shown. The data are expressed as the means ± S.D. ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$.

10, compared with FG-Ad-shLuc, approximately 10% increase in the knockdown efficiency was found for AdΔVR-shLuc. On the other hand, HD-Ad-shp53 showed a 37.3% increase in the knockdown efficiencies in WI38 cells, compared with FG-Ad-shp53 (Fig. 4A), although there was an approximately 2-fold increase in the copy number of sip53 following transduction with HD-Ad-shp53, compared with FG-Ad-shp53 (Fig. 2C). It might be difficult to largely improve the knockdown efficiency of shLuc used in this study by deletion of VA-RNA gene from the Ad vector genome because sufficiently efficient knockdown of luciferase gene was obtained even by FG-Ad-shLuc. By contrast, knockdown of p53 by FG-Ad-shp53 was less efficient than that of luciferase by FG-Ad-shLuc. Therefore, deletion of VA-RNA gene more largely elevated the knockdown efficiencies of shp53. A previous study demonstrated that overexpression of Ago2 more dramatically improved the knockdown efficiency of less efficient siRNA than high potent siRNA (Diederichs et al., 2008). The degree of increase in the knockdown efficiencies of HD-Ad-shRNA would be different depending on target genes, transduced cells, and/or knockdown capacity of shRNA.

5. Conclusions

In summary, we have demonstrated that VA-RNAs expressed from the Ad vector genome inhibited efficient knockdown by Ad vector-mediated RNAi. To the best of our knowledge, this is the first study demonstrating the effects of Ad vector-derived VA-RNAs on Ad vector-mediated RNAi. HD-Ad and AdΔVR vectors are highly promising as a platform for shRNA-mediated knockdown and to provide important clues for the improvement of RNAi-mediated knockdown systems using Ad vectors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.09.021.

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